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A screen for mutations in zebrafish that affect myelin gene expression in Schwann cells and oligodendrocytes

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Abstract

Myelin is the multi-layered glial sheath around axons in the vertebrate nervous system. Myelinating glia develop and function in intimate association with neurons and neuron–glial interactions control much of the life history of these cells. However, many of the factors that regulate key aspects of myelin development and maintenance remain unknown. To discover new molecules that are important for glial development and myelination, we undertook a screen of zebrafish mutants with previously characterized neural defects. We screened for *myelin basic protein* (*mbp*) mRNA by in situ hybridization and identified four mutants (*neckless, motionless, iguana* and *doc*) that lacked *mbp* expression in parts of the peripheral and central nervous systems (PNS or CNS), despite the presence of axons. In all four mutants electron microscopy revealed that myelin-forming glia were present and had formed loose wraps around axons but did not form compact myelin. We found that addition of exogenous retinoic acid (RA) rescued *mbp* expression in *neckless* mutant embryos, which lack endogenous RA synthesis. Timed application of the RA synthesis inhibitor DEAB to wild type embryos showed that RA signalling is required at least 48 h before the onset of myelin protein synthesis in both CNS and PNS.

Keywords: Myelin; Zebrafish; Schwann cells; Oligodendrocytes; Genetic screen; Retinoic acid

Introduction

Myelin is a specialization of vertebrate nervous systems that enables rapid saltatory conduction of action potentials. Physically, it takes the form of a multi-layered glial sheath around axons. In the peripheral nervous system (PNS), it is produced by Schwann cells and in the central nervous system (CNS) by oligodendrocytes.

In all vertebrates that myelinate, including fish, Schwann cells develop from neural crest-derived precursors, which associate with and proliferate along axonal tracts that grow out from the neural tube and peripheral ganglia (reviewed in Jessen and Mirsky, 2005). Schwann cell precursors extend processes that envelop axon bundles and progressively segregate and subdivide them. Ultimately, each myelinating Schwann cell ensheaths a single axonal segment, then elaborates a multi-layered myelin sheath that gradually becomes compacted. In the CNS, oligodendrocyte progenitors (OLPs) are generated by neuroepithelial precursors in parts of the ventricular zone (VZ) of the embryonic neural tube. They proliferate and migrate widely from their sites of origin before associating with axons and differentiating into oligodendrocytes, which elaborate myelin sheaths round single or multiple axons. In the spinal cord, the great majority of OLPs are formed from a specialized part of the ventral VZ called pMN, which also generates motor neurons (reviewed by Richardson et al., 2006).

The stages of Schwann cell and oligodendrocyte development can be delineated by the use of molecular markers that characterize the different stages of development and differentiation, including myelination (Jessen and Mirsky, 2005; Richardson, 2001). In terrestrial vertebrates and teleost fish, cells of both Schwann cell and oligodendrocyte lineages express the HMG box protein SOX10 throughout their development and

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subsequent differentiation (Dutton et al., 2001; Kuhlbrodt et al., 1998; Park et al., 2002). A transcription factor that is specifically expressed in early Schwann cell development is the winged helix protein FOXD3. In mice, *FoxD3* is preferentially expressed in developing neural crest derivatives and its zebrafish homologue, *foxD3*, has been observed in glial cell precursors associated with PNS axonal tracts. Transgenic *foxD3-GFP* zebrafish have been used to follow the co-migration of myelinating glial cells and axons in the lateral line (Gilmour et al., 2002; Kelsh et al., 2000; Labosky and Kaestner, 1998).

In mammals, birds and teleost fish, oligodendrocyte precursors (OLPs) can be identified, prior to and during migration, by expression of the transcription factors *Olig2* and/or *Olig1* (Lu et al., 2000; Park et al., 2002; Takebayashi et al., 2000; Zhou et al., 2000). *Olig* genes are required for the generation of OLPs since compound disruption of *Olig1* and *Olig2* results in a complete lack of oligodendrocytes throughout the CNS. *Olig2* null mice or zebrafish treated with *olig2*-suppressing morpholino oligonucleotides show deficiencies of both motor neurons and OLPs, indicating that these two cell types share common precursors in the spinal cord and brainstem (Lu et al., 2002; Park et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002).

In teleost fish, myelination in both PNS and CNS is characterized by upregulation of myelin proteins such as myelin protein zero (Mpz), myelin basic protein (Mbp), and orthologs of the DM20 isoform of proteolipid protein (Plp1a/b) (Brosamle and Halpern, 2002; Schweitzer et al., 2003, 2005). The process has been well characterized in zebrafish. Upregulation of *mbp, mpz* and *plp1b* begins in a few oligodendrocytes in the hindbrain at 48 hpf. At this stage, *mbp* expression is also detectable in the anterior-most Schwann cells of the lateral line. However, neither *plp1b* nor *mpz* are expressed in the PNS at this stage. The number of myelin-forming oligodendrocytes and Schwann cells increases gradually and in a rostro-caudal gradient over the next few days. Axons ensheathed by loosely wrapped glial processes can be detected in the ventral hindbrain and the lateral line by 4 days post-fertilization and compact myelin is observed by 7 days in the hindbrain, lateral line and optic nerves (Brosamle and Halpern, 2002; Schweitzer et al., 2003).

Myelin differentiation in both the CNS and PNS is regulated by interactions between Schwann cell precursors/OLPs and neurons. In rodents, neuronal β-neuregulin, signalling via *Erb2* and *Erb3* receptors, is required for myelination and regulates the thickness of the myelin sheath (Dong et al., 1995; Garratt et al., 2000; Meyer and Birchmeier, 1995; Michailov et al., 2004; Taveggia et al., 2005). A recent screen for glial defects in zebrafish also implicated neuregulin signalling in Schwann cell development (Lyons et al., 2005). Effects on oligodendrocyte differentiation are less well established in vivo, but in rodents the signalling factors retinoic acid (RA) and thyroid hormone induce cultured OLPs to stop dividing and differentiate. RA acts via a *p53*-dependent pathway and directly activates the *Mbp* promoter in these cells (Noll and Miller, 1994; Pombo et al., 1999; Tokumoto et al., 2001).

To discover new molecules that are potentially important in glial development and particularly in myelination, we undertook a screen of zebrafish mutants with previously characterized neural defects. We took advantage of some of the systematic mutagenesis screens that have previously been carried out in zebrafish. Early screens focussed on morphologically obvious defects in early neural development (Granato and Nusslein-Volhard, 1996). More recent screens have searched for more subtle effects, for example on the number or identity of immunohistochemically distinct classes of neuron (Guo et al., 1999). We screened a subset of both these types of mutant for associated defects in Schwann cell and oligodendrocyte development and differentiation during larval development, focussing particularly on Schwann cells of the posterior lateral line (PLL) nerve and oligodendrocytes in the spinal cord. We screened by in situ hybridization for defects in either temporal or spatial expression of *mbp* mRNA. Both Schwann cells and oligodendrocytes express *mbp* highly at the onset of myelination and it is easily visualized in the PLL and spinal cord (Brosamle and Halpern, 2002; Lyons et al., 2005). Our screen of 39 mutant lines identified two loci (*neckless* and *motionless*) in which there was no *mbp* expression in posterior lateral line (PLL) glia, despite the presence of axons, and *mbp* expression was also affected in a proportion of CNS oligodendrocytes. In 2 other mutants (*iguana* and *doc*), *mbp* expression was restricted to the most anterior glial cells of the PLL. In all of these mutants electron microscopic (EM) analysis showed that Schwann cells were present and had begun to wrap axons but had not formed fully differentiated myelin. The *neckless* gene product is required for RA synthesis; we used RA inhibitors and phenotypic rescue by application of exogenous RA to show that RA signalling is required at an early stage of nervous system development, long before the onset of myelin gene expression and even before the formation of overt glial-specific precursors (Schwann cell precursors or OLPs).

Materials and methods

Tissue preparation

Zebrafish specimens (*Danio rerio*) in different stages of development were maintained according to standard procedures at the Zebrafish Group, Anatomy Department, University College London, UK. Embryos, larvae and juvenile specimens were collected from pair matings, raised at 28°C with a 14-h/10 h light–dark cycle. They were staged according to hours post-fertilization (hpf) and morphological criteria (Kimmel et al., 1995).

To prepare sections larvae were anaesthetized with 0.3% tricaine methane sulfonate, MS22 (Sigma) and then fixed in fresh fixative overnight. To cryoprotect, tissue was transferred to 20% sucrose in PBS and left at 4°C for a further 24 h. Samples were then embedded in Tissue-Tek OCT compound (Agar Scientific Ltd., UK) and frozen slowly on dry ice. Frozen 10–15 μm sections were cut using a cryostat microtome.

Whole mount RNA in situ hybridization

Digoxeginin-labeled RNA probes were synthesized following the manufacturer's instructions (Roche Molecular Biochemicals). Whole-mount RNA in situ hybridization was performed as previously described (Thisse et al., 1993; Xu et al., 1994).

Larvae were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) at pH 7.5. After incubation in PBS + 0.1% Tween 20 (PBT), larvae were permeabilized by proteinase K treatment (40 μg/ml in PBT, 1 h for larvae at 5 dpf), postfixed in 4% paraformaldehyde, and washed extensively followed by incubation in hybridization mix (50% deionized formamide, 5× SSC, 0.1% Tween 20, torula RNA 5 mg/ml, heparin 50 μg/ml, pH 6.0) for 3 h at 65°C. Probe hybridization was carried out over night at 65°C. Unbound probe was removed by a series of washes. Larvae were blocked for 3 h in 2% Blocking Reagent (Roche Molecular Biochemicals) in MABT solution (Maleic acid (0.1 M), NaCl (0.15 M), pH 7.5 plus 0,1% Tween 20). Anti-digoxygenin Fab fragments coupled to alkaline phosphatase (Roche Molecular Biochemicals) were used to detect hybridized probe using BM Purple AP Substrate (Roche Molecular Biochemicals). Larvae were transferred into glycerol and photographed using a Polaroid PDMC-3 camera.

The RNA probes used included *mbp*, *plp1b*, *mpz* and *foxD3* prepared from EST clones fj45g01, fj43c04, fj33a07 and fk47f08 (Brosamle and Halpern, 2002; Kelsh et al., 2000). Probes for *sox10* (Dutton et al., 2001) and *olig2* (Park et al., 2002) were synthesized from cloned PCR products amplified from zebrafish genomic DNA using the primer pairs 5′-ACC GTG ACA CAC TCT ACC AAG ATG ACC-3′/5′-CAT GAT AAA ATT TGC ACC CTG AAA AGG-3′ and 5′-GCT TCA TCT CCT CCA GCG AGG-3′/5′-AAA CTG AGA GCG CAC TGA ACC-3′.

Immunohistochemistry

For whole mount immunohistochemistry, larvae were fixed in 10% trichloroacetic acid (TCA) for 3 h, permeabilized in 0.25% trypsin in PBT on ice, extensively washed and stored in PBT. After blocking (1% DMSO, 10% goat serum, 0.8% Triton X-100 in PBT) for 1–3 h, antibodies for acetylated tubulin (Sigma) were applied overnight in the same solution. Antibody binding was detected using either horseradish peroxidase, fluorescein isothiocyanate (FITC) or Alexa 568 conjugated secondary antibodies. For sections embryos were fixed and prepared as for in situ hybridization. Mouse monoclonal antibody for Isl1 (Developmental Studies Hybridoma bank) was applied overnight in PBT and antibody binding detected using rhodamine conjugated anti-mouse secondary antibodies (Roche Molecular Biochemicals). Samples were examined using either a Nikon Eclipse 800 photomicroscope or a Leica confocal microscope.

Electron microscopy

For electron microscopy, larvae were fixed overnight in 6% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4, washed in 0.1 M cacodylate and postfixed in 2% osmium tetroxide. After washing, larvae were incubated in 2% uranyl acetate solution for 1 h. After several rinses in water, larvae were dehydrated, followed by epoxy propane, infiltration and embedding in resin mixture (Agar scientific Ltd.). Ultrathin sections were examined in a JEOL 1010 electron microscope.

Retinoic acid and DEAB treatment

To rescue *nls* mutants by application of RA, eggs were incubated from tail bud stage (10 hpf) to 16 somites (16 hpf) or from 16 somites to 24 somites (19 hpf) in E3 medium containing 0.01 M all *trans* RA (Sigma R2625). This medium was prepared by diluting a 10 M stock solution of all-*trans* RA in DMSO 1:1000 in E3. At the end of the treatment window, the embryos were rinsed several times in E3 before replacing the RA-containing medium with fish water. DEAB (4-diethylaminobenzaldehyde; Fluka) was applied at a concentration of 10^{-5} M in E3 medium from a 10^{-2} M stock in DMSO. Control embryos were treated for the same time periods with DMSO in E3.

Results

Screening neurological mutants for myelination defects

We screened 39 mutants with established neurological, neural crest or behavioral phenotypes for myelination defects in the PLL (Table 1). Our rationale for this was that, given the shared developmental origins of neurons and glia and the extensive interactions required for normal myelination, mutations in many genes that affect neuronal development might also be expected to impact glial development. For the first stage of the screening process, we used in situ hybridization of whole mount embryos/larvae to identify mutants with abnormal *mbp* expression in the developing PLL. *Mbp* is the first myelin protein gene to be expressed by Schwann cells in the PLL (Brosamle and Halpern, 2002). At 2.5–3 dpf *mbp* transcripts can be detected in the anterior-most glial cells of the PLL, immediately posterior to the PLL ganglion (Fig. 1A). *Mbp* expression gradually spreads to more posterior glial cells and by 5 dpf can be detected throughout the PLL (Figs. 1B–D). Other PNS myelin protein genes including *plp1b* (Fig. 1E) and *mpz* (Schweitzer et al., 2003) show a similar anterior-to-posterior sequence of activation in the PLL. We screened for *mbp* expression in the PLL between 3 and 5 dpf depending on mutant viability (the majority of the mutants die around 4–5 dpf). Screening late in the myelination process allowed us to detect effects on the differentiation of myelinating glia as well as the specification, proliferation and survival of their precursors.

Of the 39 mutants screened, 25 had normal *mbp* expression in the PLL. In 9 mutants the line of *mbp* expression followed an abnormal path, in 4 mutants *mbp* was not expressed in the PLL at all and in 2 mutants it was restricted to the anterior-most glial cells. For the second stage of the screening process we used the axonal marker acetylated tubulin to determine whether the abnormal *mbp* expression we had observed might simply reflect axonal abnormalities in the PLL of these mutants.

In wild type fish the PLL nerve runs along the horizontal myoseptum from the PLL ganglion to the tail (Fig. 1F). By 4 dpf, the mechanosensory neuromasts of the PLL have migrated away from the myoseptum to slightly more ventral locations and axons can be seen branching off from the main PLL nerve bundle to maintain innervation of the neuromasts. In all 9 mutants in which *mbp* expression followed an abnormal path, PLL axons were found to be misrouted in the same way. We concluded that in these mutants the underlying defect was in axonal pathfinding and discarded them from the study. In all 6 mutants showing truncated/absent *mbp* expression in the PLL the axons were clearly present in all larvae that hatched. In these mutants, the defect in *mbp* expression could therefore not be accounted for by the nerve simply failing to form.

For the third and final stage of the screening process, we used electron microscopy (EM) analysis to determine whether morphologically differentiated myelinating glia were present in the PLL. In wild type fish Schwann cells begin to enwrap axons before 72 hpf (Levavasseur et al., 1998) and by 5 days multiple loose wraps can be seen by EM (Figs. 1G, H). Differentiating Schwann cells enwrapping the PLL axons were present in all four of our *mbp* expression mutants.

Doc and iguana

Mutations in *doc* (*doc*) have cell-autonomous effects on notochord development and are associated with failure to

Table 1
Zebrafish mutants screened for defects in *mbp* expression in the PLL

Locus	Allele	Gene	Comments on PLL phenotype	Other phenotypes/references
acerebellar	ti282	fgf8	Faint *mbp* expression with small gaps	Brain and midline defects (Brand et al., 1996; Reifers et al., 1998; Trowe et al., 1996; Whitfield et al., 1996)
akineto	u45		Normal	Non-motile
beamter	tm98		Normal	Hindbrain neurogenesis (Julich et al., 2005)
casanova	a56b	sox32	Faint *mbp* expression	Endoderm development (Chen et al., 1996; Dickmeis et al., 2001)
chinless	b146		Faint *mbp* expression with gaps	Neural crest defects (Schilling et al., 1996)
cyclops	m294	Nodal-related 2	Normal	CNS defects (Brand et al., 1996)
dackel	to79c	exostoses (multiple) 2 (ext2)	Normal	Abnormal fin, retinotectal projection (Karlstrom et al., 1996; Whitfield et al., 1996)
eisspalte	ty77e		Normal	Dented hindbrain (Jiang et al., 1996; Trowe et al., 1996)
flotte lotte	ti262c		Normal	Forebrain defects (Heisenberg et al., 1996)
hammerhead	to16		Normal	Forebrain defects (Piotrowski et al., 1996)
hands off	s40	hand2	Normal	Jaw and fin defects (Beis et al., 2005)
heart and soul	m129	aPKC	Faint *mbp* expression	Reduced brain ventricles (Horne-Badovinac et al., 2001; Schier et al., 1996)
headless	m881	tcf7l1a	Normal	Brain defects (Kim et al., 2000)
hoover	tn213		Normal	Jaw defects (Piotrowski et al., 1996)
masterblind	tm213	axin	*Normal*	Forebrain defects (Heisenberg et al., 1996, 2001)
miles apart	m93	edg5	Faint *mbp* expression with gaps	Heart defects (Chen et al., 1996; Kupperman et al., 2000)
mind bomb	m132	RING ubiquitin ligase	Normal	Neurogenesis and brain defects (Itoh et al., 2003; Jiang et al., 1996; Schier et al., 1996; van Eeden et al., 1996)
monorail	v53a		Normal	Floorplate development (Chen et al., 1996; Norton et al., 2005)
one eyed pinhead	tz57	oep	Faint *mbp* expression in caudal region	Floorplate defects (Schier et al., 1996; Stemple et al., 1996; Zhang et al., 1998)
parachute		cdh2	Normal	Brain and eye defects (Jiang et al., 1996; Lele et al., 2002; Masai et al., 1997; Odenthal et al., 1996; Trowe et al., 1996)
silberblick	u148	wnt11	Normal	Forebrain defects (Heisenberg and Nusslein-Volhard, 1997; Heisenberg et al., 1996; Piotrowski et al., 1996)
sucker	tf216b	endotelin 1	Normal	Jaw defects (Miller et al., 2000; Piotrowski et al., 1996)
schmalspur	m786	foxh1	Normal	Brain and ventral neurectoderm defects (Chen et al., 1996; Piotrowski et al., 1996; Pogoda et al., 2000; Schier et al., 1996; Stemple et al., 1996)
trilobite	m209	Vangl2	Low level *mbp* expression	Notochord defects (Jessen et al., 2002; Stemple et al., 1996)
bashful	u13	lama1	Line deviates and loops away from myoseptum	Notochord and brain defects (Jiang et al., 1996; Schier et al., 1996; Stemple et al., 1996)
biber	tb8		Expression in multiple thin branches along course of myoseptum	Severe pattern defects (Hammerschmidt et al., 1996a)
dino	m84	chordin	Line deviates and loops away from myoseptum	Ventralised embryos (Hammerschmidt et al., 1996b; Odenthal et al., 1996; Schulte-Merker et al., 1997)
faust	am36a	gata 5	Expression normal up to somites 3–5 then fades and deviates from myoseptum	Cardiac defects (Chen et al., 1996; Reiter et al., 1999)
floating head	n1	flh	Line deviates and loops away from myoseptum	Notochord defects (Odenthal et al., 1996; Talbot et al., 1995)
slow muscle omitted	b641	smo	Line deviates and loops away from myoseptum	Floorplate and ventral neural tube defects (Varga et al., 2001)
spade tail	b104	Tbx16	Line deviates and loops away from myoseptum Low level mbp expression	Notochord defects (Eisen and Pike, 1991; Griffin et al., 1998; Odenthal et al., 1996)
U-boot	tp39	Prdm1	Line deviates and loops away from myoseptum	Neural crest and locomotion defects (Baxendale et al., 2004; Granato et al., 1996)
you too	ty119a	Gli2	Line deviates and loops away from myoseptum	Neural tube defects (Dickmeis et al., 2001; Karlstrom et al., 1996; Piotrowski et al., 1996; van Eeden et al., 1996)
iguana	tm79a	dzip1	No *mbp* expression beyond first 5 somites	Midline defects (Chen et al., 1996; Sekimizu et al., 2004; Wolff et al., 2004)
doc	tt258		No *mbp* expression beyond first 5 somites	Notochord and locomotion defects (Chen et al., 1996; Granato et al., 1996; Odenthal et al., 1996; van Eeden et al., 1996)
neckless	i26	aldh1a2	No *mbp* expression in PLL but present in other parts of the PNS	Hindbrain patterning and neuronal defects (Begemann et al., 2001,2004)
no fin		aldh1a2	As *neckless* but incompletely penetrant	Hindbrain patterning defects (Grandel et al., 2002)
motionless	m807		No *mbp* expression in PNS or CNS	Forebrain and neuronal defects (Guo et al., 1999)
otter	ta76b		As *motionless*	Forebrain and neuronal defects (Guo et al., 1999)

All mutants were ENU induced with the exception of *chinless* (γ irradiation) and floating head (spontaneous). References to other relevant phenotypic effects (neural development, locomotion etc).

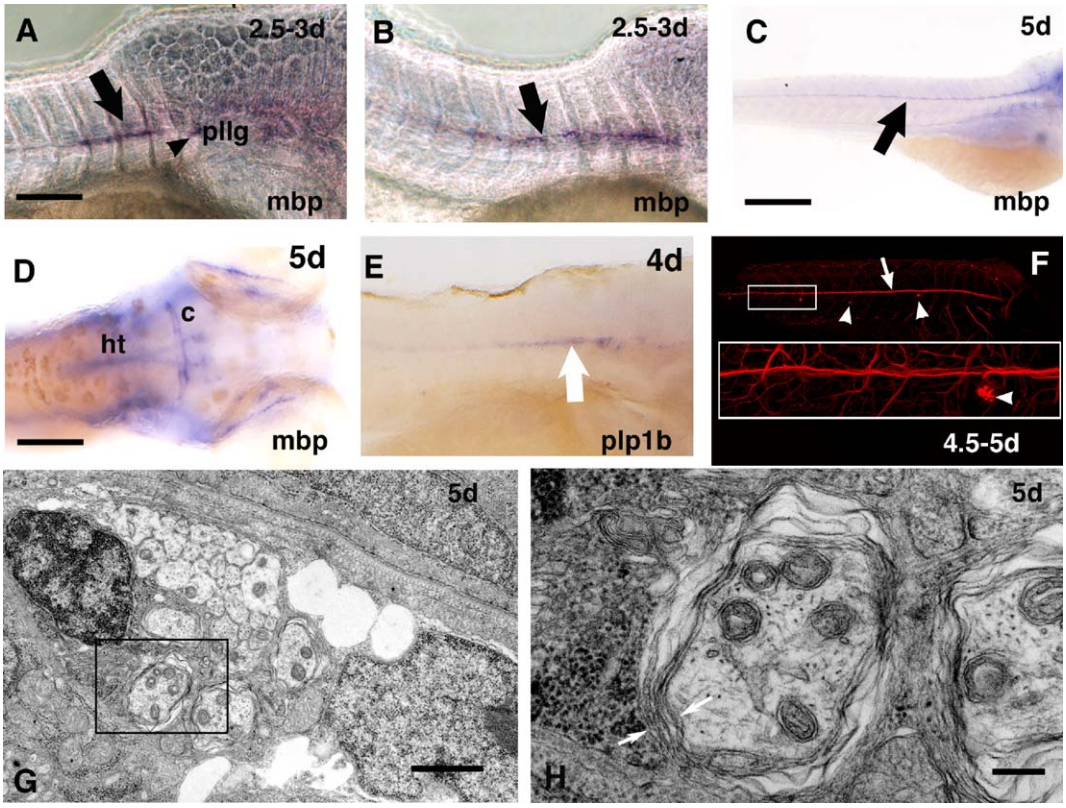

Fig. 1. Myelination and neuronal differentiation in the PLL. (A–F) show whole mount preparations of 2.5–5 dpf zebrafish. (A) *Mbp* expression begins around 2.5 dpf in myelinating glial cells (arrow) immediately posterior (arrowhead) to the posterior lateral line ganglion (pllg). (B) Expression is activated in progressively more posteriorly situated cells as time progresses. By 3 dpf *mbp*-expressing cells (arrow) extend 5–7 somites posterior to the pllg. (C) By 5 dpf *mbp* (arrow) is expressed all along the PLL. (D) *Mbp* expression in the anterior lateral line and the hindbrain at 5 dpf. c—commissure. ht—hindbrain tract. (E) *Plp1b* expression in the PLL (arrow) begins around 4 dpf. (F) PLL axons (arrow) at 5 dpf labeled with anti-acetylated tubulin. Higher power view of inset shows axons extending ventrally to innervate individual neuromeres (arrowheads). Panels G, H are electron micrographs of the PLL of a 5 dpf larva showing a transverse section through the PLL. (H) High power view of inset in panel G. Note loose wraps (arrowheads) around the large diameter axon. Scale bars in panels A, B and inset in panel F 5 μm; in panel C 20 μm; in panels D–F 10 μm; in panel G 1 μm; in panel H 200 nm.

maintain expression of the zebrafish T-brachyury homologue *no tail* (Odenthal et al., 1996). *Iguana* (*igu*) is a member of the midline group of mutants and shows the characteristic curved body shape and defects in cell fate specification in the ventral neural tube and somites (Brand et al., 1996). It has been cloned and codes for a novel intracellular regulator of hedgehog signalling Dzip1 (Sekimizu et al., 2004; Wolff et al., 2004).

Doc and *igu* mutants show very restricted *mbp* expression in the PLL (Figs. 2A, B). Even at 5 dpf *mbp* can only be detected in the anterior-most segments, although by this stage the PLL nerve extends almost the full length of the fish in both these mutants (Figs. 2C, D). In *doc* mutants, EM analysis reveals that morphologically differentiating myelinating glial cells are still present in posterior segments, in which there is no detectable *mbp* (Figs. 2E, F).

Motionless/otter

Motionless (*mot*) is likely to be allelic with *otter* (*ott*), a previously described mutant with ventricular defects. *Mot* mutants also show defects in certain classes of CNS neurons and anterior commissure formation is also severely disrupted (Guo et al., 1999).

Mot and *ott* mutants die around 4 dpf and show no expression of *mbp* in either the PNS or the CNS at any stage (Figs. 3A, B). We could detect no differences between the myelination phenotypes of *mot* and *ott* and further analysis has focussed exclusively on *ott*.

Ott mutants lack *plp1b* as well as *mbp* expression, although *mpz* is detectable in the CNS (Figs. 3C–E). PLL axons are present and early markers for PNS myelinating glial cells, such as *foxD3*, are expressed as these cells migrate out from their origin in the PLL ganglion (Figs. 3F–J). The nerve grows out more slowly than in the wild type and never extends the full length of the fish. Despite their lack of myelin protein gene expression, PLL glial cells in the mutants appear to begin morphological differentiation and at 80 hpf are indistinguishable from wild type glial cells in EM sections (Figs. 3K, L).

We performed some further analysis of the CNS phenotype in *ott* mutants focussing on the expression of oligodendrocyte and motor neuron lineage specific markers in sections through the spinal cord. At 80 hpf no *mbp* or *plp1b*-expressing cells were present in mutant spinal cord. Occasional *mpz*-expressing cells were seen in the grey matter although not at the peripheral locations typical for differentiating oligodendrocytes in wild type fish (Figs. 4A–F). Most spinal cord

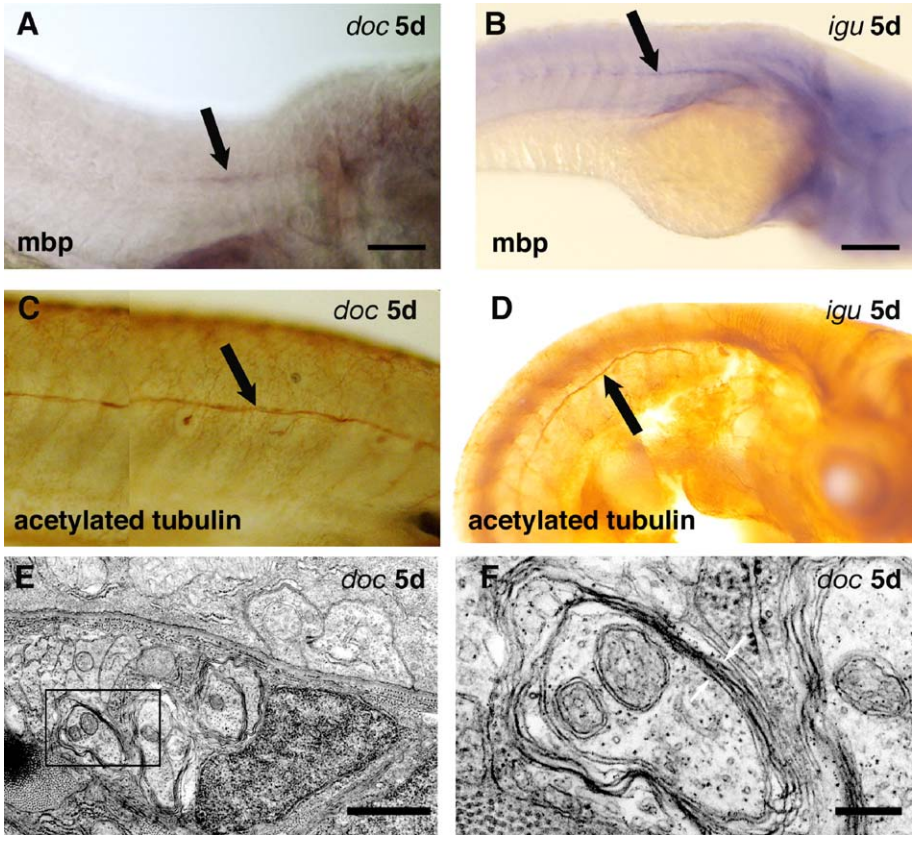

Fig. 2. PLL phenotype of *doc* and *igu* mutants. (A, B) *Mbp* expression (arrow) in 5 dpf larvae homozygous mutant for *doc* and *igu* respectively. Expression is restricted to the anteriormost section of the PLL. (C, D) Anti-acetylated tubulin staining (arrow) of PLL axons in panel C *doc* and panel D *igu* mutants. A PLL nerve extends to the posterior segments (arrows) of the fish in both mutants. (E, F) Electronmicrographs showing a transverve section of the rostral (*mbp* expressing) PLL in *doc* mutants. Axons ensheathed by loosely wrapped myelin (arrows) are clearly present in the mutant nerve. Scale bars in panels A and C, 5 μm; in panels B and D, 10 μm; in panel E, 1 μm; in panel F, 200 nm.

oligodendrocytes originate from the ventral *olig2*-expressing domain of the VZ that earlier gives rise to motor neurons (Park et al., 2004). We analyzed the expression of *olig2* and the early motor neuron marker Islet1 (Isl1) in *ott* mutants (Figs. 4G–J). At 80 hpf, the distribution of Isl1-expressing cells appeared normal but *olig2* was still restricted to the ventricular zone, whereas in wild type fish it could also be seen in the peripheral cells, which are thought to represent migrating OLPs (Park et al., 2002). As in the PNS, EM analysis of spinal cord identified differentiating glial cells had begun to loosely wrap axons (Figs. 4K, L).

Neckless/no fin

Both *neckless* (*nls)* and *no fin* (*nof)* mutations disrupt the zebrafish homologue of the RA synthesis enzyme *Aldh1a2* (Begemann et al., 2001; Grandel et al., 2002). This enzyme catalyzes the final step in RA synthesis from vitamin A and is expressed during early embryogenesis. In zebrafish, mutations in *aldh1a2* act non-cell-autonomously to cause rhombomeres 5–7 to expand and affect the differentiation of catecholaminergic and brachiomotor neurons in the hindbrain. A recent analysis (Begemann et al., 2004) of *nls* mutants prior to myelinogenesis (48 hpf) showed that in a proportion (30%) of embryos, expression of the axonal marker Tag-1 in the PLL nerve was attenuated, suggesting that in these embryos the axons fail to extend past the level of the first somite. Both the size of the PLL ganglion and the thickness of the axonal bundle were reduced in all embryos. A significant fraction of mutant embryos die shortly after this stage of development.

We found that *mbp* expression is absent from the PLL of *nls* and *nof* mutants at 5 dpf but present at normal levels in the anterior lateral line and all other regions of the PNS and CNS (Fig. 5A). In all mutants that survive to 5 days, the PLL nerve was present and extended the entire length of the fish. We found that at least 50% of mutant fish fail to hatch and infer that this fraction includes the 30% in which axons fail to extend (Begemann et al., 2004). However, we observed that, although the nerve itself appeared normal in mutants that survived for 5 days, the branches that innervate individual neuromeres were abnormally short (Fig. 5B). In contrast to the *nls* mutants, *nof* mutants displayed a variable *mbp* phenotype—only ~40% (17/44) mutants had abnormal *mbp* expression in the PLL and, in ~12% of those that did, *mbp* expression was normal on one side of the fish and completely absent on the other (Figs. 5C, D). Since the *nls* phenotype was relatively invariant, we used this mutant for all subsequent analysis of the effects of *aldh1a2* disruption on PLL development.

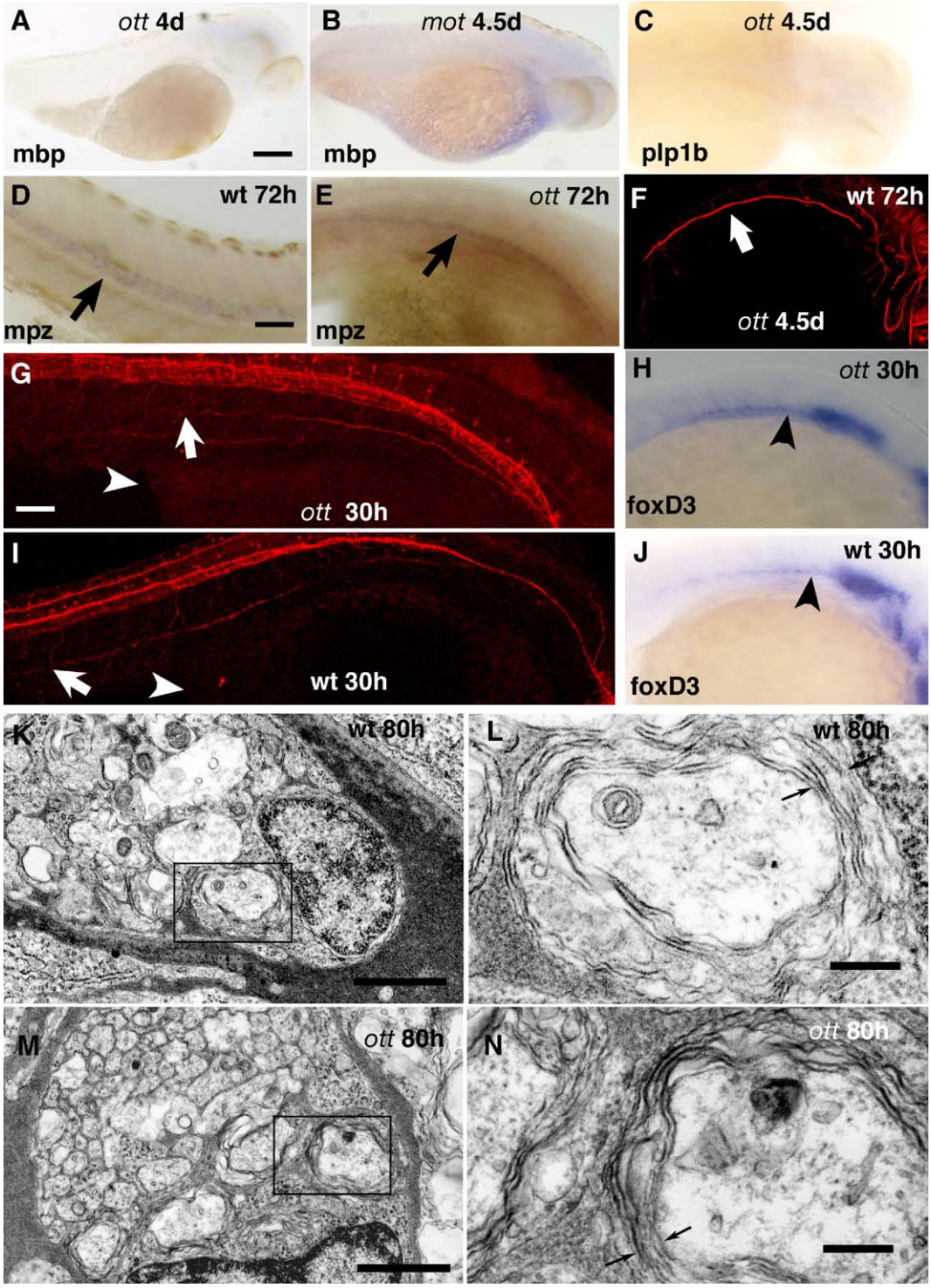

Fig. 3. PLL phenotype of *mot/ott* mutants. (A–C) Myelin protein expression in *ott* and *mot* mutants. Both mutants show a complete lack of *mbp* and *Plp1b* expression. (D, E) *Mpz* expression in the CNS (arrows) of wild type (D) and *ott* (E) embryos at 72 hpf. *Mpz* is expressed at reduced levels in *ott* embryos. (F) Anti-acetylated tubulin staining of the PLL in *ott* mutants. The axons (arrow) are present but do not extend the whole length of the fish. (G–J) Early development of the PLL nerve and glia precursors. At 30 hpf, axons of the PLL nerve (arrows)of *ott* mutants (G) have extended less far than in the wild type. Arrowhead—caudal end of yolk sac (I). Prior to myelination, early glial expression (arrowheads) of *foxD3* in both mutant (H) and wild type (J) fish. (K–N) Electronmicrographs of transverse sections through the PLL nerve of wild type (K, L) and *ott* mutant (M, N) fish 80 hpf. The larger diameter axons are ensheathed by loosely packed myelin (arrows) typical of this early stage of development. Scale bars in panels A–C 10 μm; in panels D–F, H and J 5 μm; in panels G and I 3 μm; in panel E 1 μm; in panels K and M; in panels L and N 200 nm.

Plp1b expression can be detected in the PLL of *nls* mutant fish at reduced levels compared to wild type (Figs. 5E, F). EM analysis of 5-day-old mutants also showed cells enwrapping the axons of the PLL which were morphologically indistinguishable from wild type myelinating glia at this stage of normal development (Figs. 5G, H).

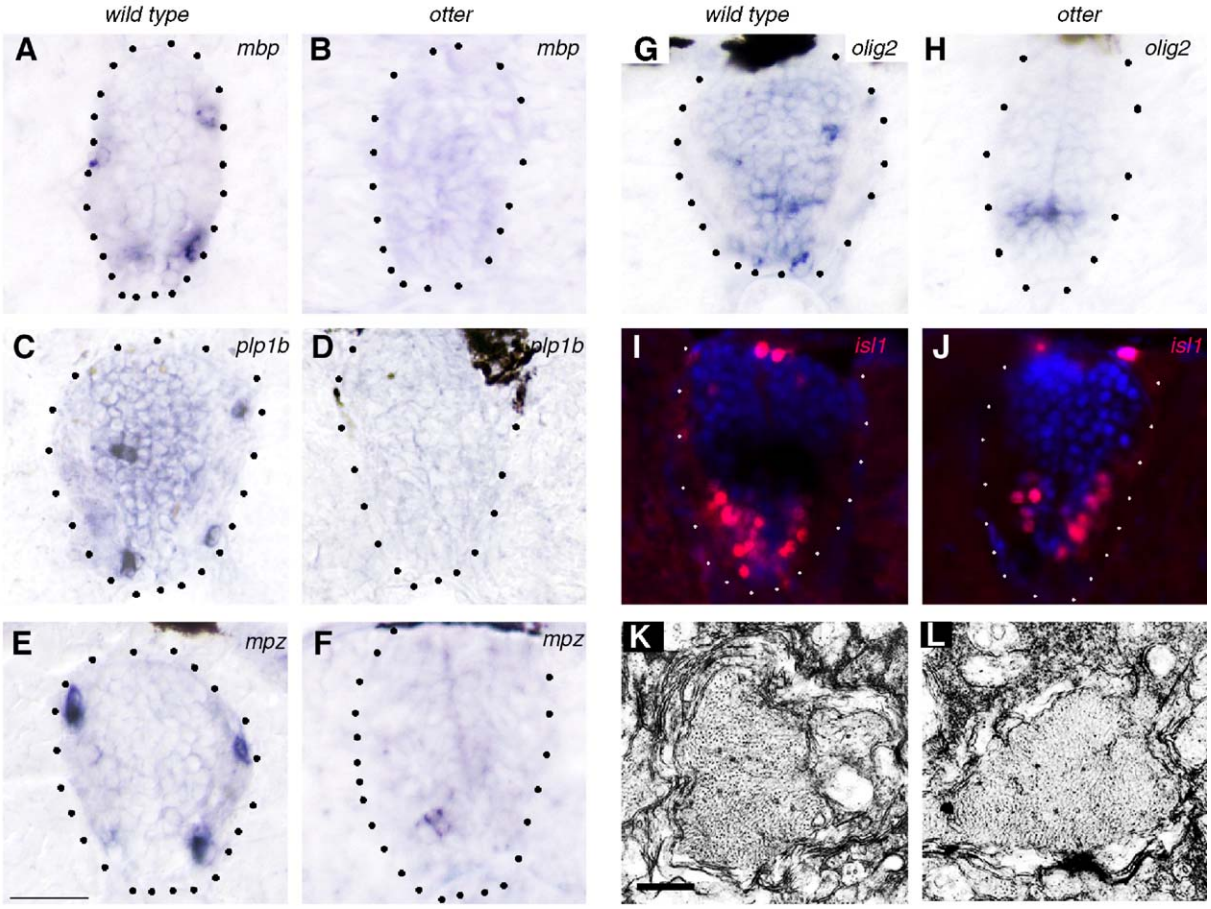

Fig. 4. CNS phenotype of *ott* mutants. Transverse sections through the spinal cord of 80 hpf larvae. (A–F) Myelin protein expression in wild type (A, C, E) and *ott* (B, D, F) mutant fish. (A, B) *mbp* is not expressed in *ott* mutants. (C, D) *Plp1b* is not expressed in *ott* mutants. (E, F) *Mpz* is weakly present in some grey matter cells in *ott* mutants. (G, H) In wild type fish, *olig2*-expressing cells have migrated to the periphery by 80 hpf (G) whereas *olig2* expression is still restricted to the ventral ventricular zone in *ott* mutants (H). (I, J) The distribution of cells expressing the motor neuron marker i*sl1* is similar in wild type (I) and *ott* mutant (J) fish. (K, L) Electronmicrographs of spinal cord axons ensheathed by loosely wrapped immature myelin in wild type (K) and *ott* mutant (L) fish. Scale bars in panels K and L, 200 nm.

An effect on *mbp* expression is also seen in the spinal cord of *nls* mutants. We observed a complete lack of *mbp* expression in spinal cord oligodendrocytes in ~85% (41/47) of 5-day-old *nls* larvae, although the oligodendrocyte precursor marker *sox10* is expressed normally (Figs. 6A–C). Cells with a similar morphology to wild type differentiating oligodendrocytes can be seen by EM (data not shown). A similar lack of *mbp* expression is seen in wild type fish treated with the RA synthesis inhibitor DEAB from 11 hpf (Fig. 6D).

To determine the developmental stage at which RA synthesis is required for normal *mbp* expression in both Schwann cells of the PLL and spinal cord oligodendrocytes, we used timed application of either exogenous RA (to rescue the PLL phenotype in *nls* mutants) or DEAB (to suppress RA synthesis in wild type fish). In both cases, treatment had to be applied relatively early in development; no effects on *mbp* expression were seen in the CNS of fish to which DEAB was applied later than 26 hpf (Table 2, Figs. 6E, F). Similarly, *mbp* expression was restored in the PLL of 100% of *nls* mutant fish to which RA was applied between 10 and 16 hpf but only in 33% of larvae treated between 16 and 19 hpf (Table 3, Fig. 6F).

Discussion

An advantage of zebrafish as an experimental model is the ability to conduct forward genetic screens. Screens can be performed either of newly induced mutants, bred to homozygosity following mutagenesis, or of panels of mutants that have been identified previously on the basis of some other phenotype (so-called shelf screens). Recently, at least two groups have successfully conducted de novo screens for mutations that perturb myelination and *mbp* expression. In one of these, a screen of 600 mutagenized genomes isolated eleven different genes affecting various stages of myelin development (Lyons et al., 2005). We have performed a shelf screen of 39 mutants with known CNS defects, which uncovered mutations in four loci that affect *mbp* expression in the PLL. None of these mutants had been uncovered in the de novo screens, which focused on glial cell specific phenotypes and may have rejected mutants with pleiotropic phenotypes. The relatively high success rate of our approach suggests that screening for genes involved in both neuronal and glial development would be a useful strategy to complement de novo screening efforts.

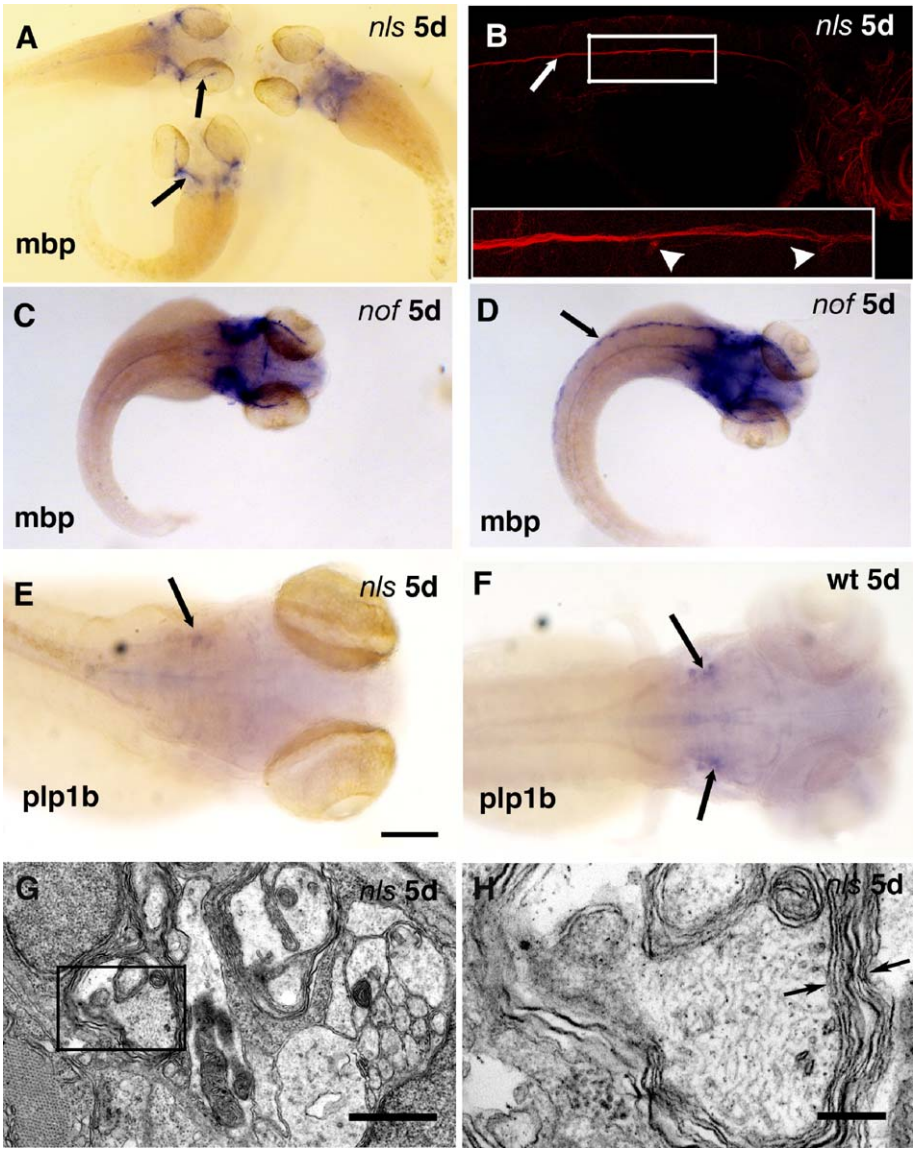

Fig. 5. PLL phenotype of *nls* and *nof* mutants. (A) 5 dpf *nls* mutant fish. Note *mbp* expression in the anterior lateral line (arrows) but absent from the PLL. (B) PLL nerve (arrow) of *nls* mutant 5 dpf stained with anti-acetylated tubulin. In the wild type, axons branch off ventrally at intervals to innervate individual neuromeres (arrowhead) but no such branching is seen in the mutant. (C, D) 5 dpf *nof* mutant fish. The *nof* phenotype shows variable expressivity and in some mutants *mpb* is expressed on one side only (arrow) as shown in panel D. Note normal *mbp* expression in the PLL on the left side (arrow). (E, F) *Plp1b* expression in *nls* mutant (E) and wild type (F) fish. *Plp1b* is detectable on both sides in the wild type and on one side of the *nls* mutant (arrow). (G. H) Electron micrographs of the PLL in *nls* mutant fish. Note loosely wrapped ensheathment by myelin (arrows) of the large diameter axons. Scale bars in panels B, E and F, 10 μm; in inset in panel B, 5 μm; in panel G 1 μm; in panel H, 200 nm.

Screening for mutants that lack *mbp*-expressing cells, we identified four loci that affect myelin protein gene expression during the differentiation stage. Layers of myelin around some axons, indicating the presence of differentiating Schwann cells and oligodendrocytes, are present in all mutant fish. The effects of *doc* and *igu* are restricted to the posterior part of the PLL while *mot* and *aldh1a2* have effects throughout the PLL. Mutations at *mot/ott* have widespread effects on neural and vascular development. All mutants die around 4 dpf, probably due to vascular defects. Previous studies show an effect on neuronal differentiation in the form of a pronounced reduction in the numbers of catecholaminergic (CA) neurons in the CNS and a failure of axon extension in those cells that do express the appropriate markers. Our observations on PLL development suggest that neural differentiation is also delayed and incomplete in the PNS. While the effects on neural differentiation are widespread, not all neurons are affected. Branchial arch-associated CA neurons and *isl1*-expressing motor neurons appear normal. By contrast, the effects on Schwann cell and oligodendrocyte differentiation are universal. No *mbp* expression can be detected in either the PNS or the CNS of *mot/ott* larvae. This suggests that the effects on myelination might be independent of the neuronal defects and possibly cell autonomous. A better understanding of the specific role played by this gene in myelinating glial cells will be possible when the locus has been cloned.

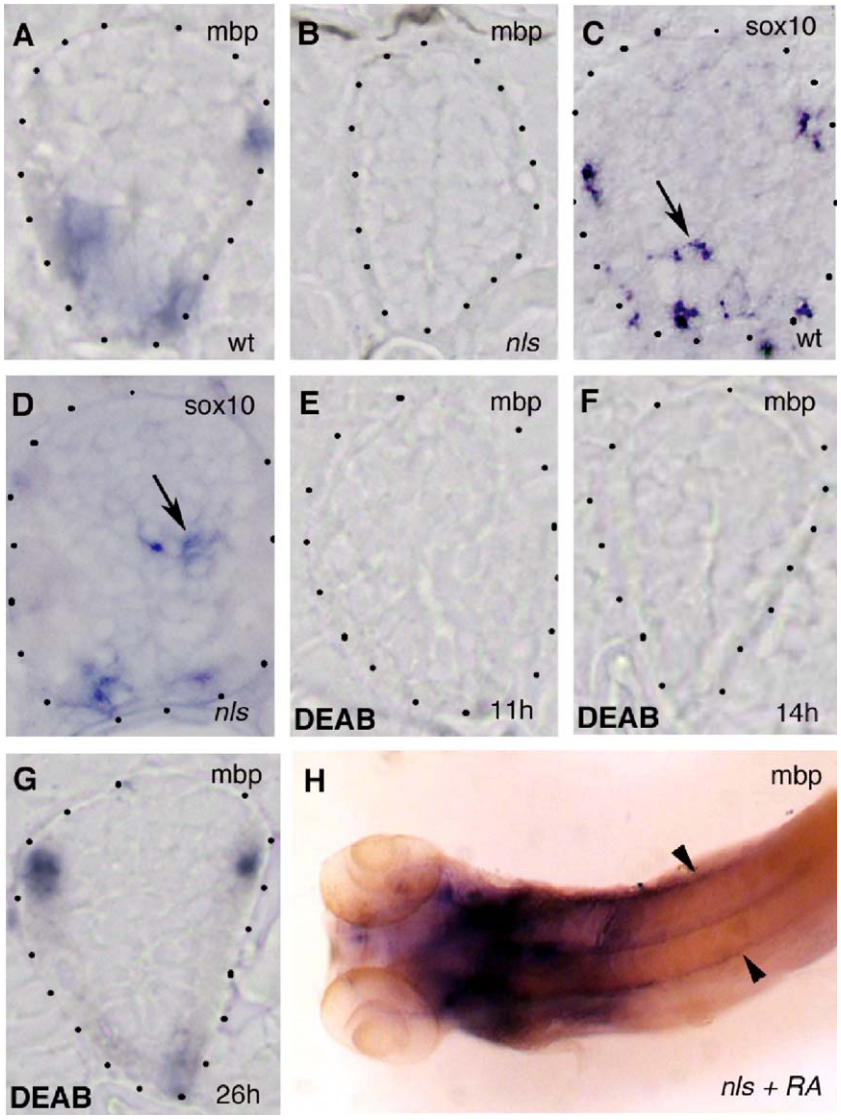

Fig. 6. (A–C) CNS phenotype of *nls* mutants at 4 dpf (A) *Mbp* expression in wild type spinal cord at 4 dpf. Differentiating oligodendrocytes are located peripherally in the white matter. (B) *Mbp* expression is absent in the great majority of *nls* mutant fish. (C) *Sox10* expression in wild type spinal cord. *Sox10* labels oligodendrocyte precursors in the grey matter (arrow) as well as differentiating oligodendrocytes in the periphery. (D) *Sox10* expression appears normal in *nls* mutants. Note labeling of oligodendrocyte precursors in the grey matter (arrow). (E–H) Effects of timed application of DEAB (D–F) and RA (G) on wild type and *nls* mutant fish respectively. (E) Effects of DEAB treatment from 11 hpf on *mbp* expression. (F) Effects of DEAB treatment from 14 hpf on *mbp* expression. (G) Effects of DEAB treatment from 26 hpf on *mbp* expression. (H) *Mbp* expression is restored in the PLL (arrowheads) of *nls* mutants treated with exogenous RA between 10 and 16 hpf.

The effects on myelination observed in *nof* and *nls* mutants are especially interesting. RA signalling has been implicated at several stages of oligodendrocyte development in higher vertebrates (Appel and Eisen, 2003) and RA has been shown to activate the *Mbp* promoter in rat optic nerve glial cell cultures (Pombo et al., 1999). However, there is less evidence that RA is required in Schwann cell development although RA synthesis enzymes and receptors are expressed in peripheral nerves (Berggren et al., 1999).

In *nls* mutant fish, the effects on the oligodendrocyte and Schwann cell lineages are surprisingly similar and specifically involve *mbp* expression as both *plp1b* and *mpz* expression can

Table 2
Effects of timed applications of the RA synthesis inhibitor DEAB on *mbp* expression in the spinal cord of zebrafish embryos and larvae

DEAB treatment	Larvae showing *mbp* expression in spinal cord oligodendrocytes at 4 dpf (%)	Total number
11 hpf	9.4	32
14 hpf	14.3	35
26 hpf	88.4	43
48 hpf	96.2	52

Table 3
Effects of timed applications of RA on *mbp* expression in the PLL of homozygous mutant *nls* zebrafish embryos and larvae

Retinoic acid treatment	Normal fins (%)	PLL mbp expression (%)	Total number
10–16 hpf	97	100	30
16–19 hpf	17	33	6

still be detected in the mutants. Given that the onset of *mbp* expression is a very late event in the development of myelin-forming cells, our finding that the requirement for RA synthesis is restricted to early stages of PNS and CNS development is unexpected. In the PNS, the earliest time that committed glial cell precursors can be identified by FoxD3 expression is at the 15-somite stage (17 hpf) (Kelsh et al., 2000) but exogenous RA applied between tailbud (10 hpf) and 14-somites (16 hpf) is sufficient to restore *mbp* expression in the PLL of *nls* mutants (Fig. 6F). Similarly, in the CNS, oligodendrocyte specific markers such as *sox10* are first expressed around 56 hpf (Park et al., 2004) but blocking RA synthesis by DEAB treatment after 26 hpf has no effect on *mbp* expression in the spinal cord (Fig. 6E). A direct effect of RA on the differentiation of myelin forming cells therefore seems very unlikely, because RA synthesis is required before the appearance of overt glial-specific precursors (Schwann cell precursors or OLPs). However, RA signalling might be required for proper development of earlier neuroglial or glial-restricted precursors, such that their ability to generate fully functional glial progeny is impaired in the mutant. As both OLPs and Schwann cell precursors divide prior to differentiation, then this interpretation implies that RA might induce some heritable change in the precursor cells that is permissive for later *mbp* expression. This could be an epigenetic modification affecting the *mbp* gene, for example.

An alternative possibility is that the primary effect of RA is on neuronal development. Unlike glial cells, neurons are present in both the PNS and the CNS during the period of RA sensitivity. PLL neurons first begin to extend axons around 16 hpf and, in the CNS, primary neurons are born around 10 hpf and secondary neurons between 16 and 25 hpf (Myers et al., 1986). Motor neuron development in the hindbrain and *mbp* expression in the CNS have similar windows of sensitivity to DEAB treatment between 14 and 17 hpf (Begemann et al., 2004; Linville et al., 2004). Early exposure to RA might be required to set in train certain aspects of neuronal differentiation that are required for the later activation of myelination in Schwann cell precursors or OLPs. Further experiments will be required to determine whether neuronal defects alone are sufficient to account for the failure to activate *mbp* expression.

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